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A Cell Culture Platform for the Cultivation of Cryptosporidium

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Keywords:	Cryptosporidium, COLO-680N, long-term cell culturing, parasites, oocysts, microscopy, western blot
Abstract:	<p>Cryptosporidium is a genus of ubiquitous unicellular parasites belonging to the phylum Apicomplexa, whose members are parasites of the gastrointestinal tract and airways. Cryptosporidium is the second largest cause of childhood diarrhoea and is associated with increased morbidity. Accompanying this is the low availability of treatment and lack of vaccines. The major barrier to developing effective treatment is the lack of reliable in vitro culture methods, in particular those which can support the complete growth of the parasite long term, while producing a high yield of the hardy oocyst stages. While numerous cell lines have been reported as maintaining the parasite, there remain no options for maintaining the parasite for longer than a week. The current cell line of choice, HCT-8, can only maintain infection for three days. Recently, our lab has successfully cultivated <i>C. parvum</i> in the oesophageal cancer derived cell line COLO-680N, and can maintain infection for several weeks. The success of this cell line was assessed with a combination of various techniques including fluorescent microscopy and qPCR. In addition, to tackle the issue of long-term oocyst production in vitro, a simple, low cost bioreactor system using the COLO-680N cell line was established, which produced infectious oocysts for 13 weeks. This chapter provides details on the methodologies used to culture, maintain and assess <i>Cryptosporidium</i> infection and propagation in COLO-680N.</p>

A Cell Culture Platform for the cultivation of *Cryptosporidium parvum*

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Keywords: *Cryptosporidium*, long-term cell culturing, COLO-680N, microscopy,

ABSTRACT

Cryptosporidium is a genus of ubiquitous unicellular parasites belonging to the phylum Apicomplexa, whose members are parasites of the gastrointestinal tract and airways. *Cryptosporidium* is the second largest cause of childhood diarrhoea and is associated with increased morbidity. Accompanying this is the low availability of treatment and lack of vaccines. The major barrier to developing effective treatment is the lack of reliable *in vitro* culture methods, in particular those which can support the complete growth of the parasite long term, while producing a high yield of the hardy oocyst stages. While numerous cell lines have been reported as maintaining the parasite, there remain no options for maintaining the parasite for longer than a week. The current cell line of choice, HCT-8, can only maintain infection for three days. Recently, our lab has successfully cultivated *C. parvum* in the oesophageal cancer derived cell line COLO-680N, and can maintain infection for several weeks. The success of this cell line was assessed with a combination of various techniques including fluorescent microscopy and qPCR. In addition, to tackle the issue of long-term oocyst production *in vitro*, a simple, low cost bioreactor system using the COLO-680N cell line was established, which produced infectious oocysts for 13 weeks. This chapter provides details on the methodologies used to culture, maintain and assess *Cryptosporidium* infection and propagation in COLO-680N.

INTRODUCTION

Cryptosporidium is an early-branching apicomplexan parasite, responsible for causing the diarrhoeal disease known as cryptosporidiosis. In humans, *Cryptosporidium parvum* and *C. hominis* are the main causative agents of cryptosporidiosis, which is the second major cause of death in under five-year-old children with diarrhoea in developing countries (Kotloff et al. 2013). In addition, infection with *Cryptosporidium* has also shown stunted growth in children (Kotloff et al. 2013, Kirkpatrick et al. 2006, Korpe et al. 2016). Despite the significance and the consequences that this parasite has in both human and animal health, until recently there were no tools to study the biology of *Cryptosporidium* in the laboratory, and most research was dependent on animal models (Sharling et al. 2010). Over the last five years there was an explosion of studies on developing *in vitro* *Cryptosporidium* tools, including CRISPR genome engineering (Vinayak et al. 2015) and methods for continuous culturing of the parasite (Morada et al. 2016, Miller et al. 2018a). In this manuscript, we will describe in technical detail, how *Cryptosporidium* can be maintained in cell culture and how to monitor its infectivity and propagation.

Earlier attempts to maintain *Cryptosporidium* in culture were hindered by problems including rapid drop in viable cell counts, incomplete life cycles along with insufficient production of sporulated infectious oocysts (Hijawi 2010, Karanis and Aldeyarbi 2011, Karanis 2017). The only reliable methods to produce infectious *Cryptosporidium* oocysts, that until recently existed, required continuous infection of immuno-suppressed animals, typically cows or sheep (Striepen 2013). New publications reported three dimension (3D) apparatuses that mimic the gut in order to accomplish cell culture-based oocyst production using HCT-8 cells together with hollow fiber technology (Morada et al. 2016) or epithelial organoids derived from human small intestine and lung (Heo et al. 2018). However, specialised equipment, know-how and very large amounts of expensive cell culture media supplements are required for such culturing.

In contrast to these complex methods, we recently found that COLO-680N oesophageal cancer cell line effectively supports the propagation of the *C. parvum* strains Moredun and Iowa (Miller et al. 2018a). Among several investigated cell lines, COLO-680N was the only one that produced substantially higher amounts of oocysts than the input oocysts, which enabled the subsequent infection of further cell cultures. In contrast, the

1
2
3 supernatants of *Cryptosporidium*-infected HCT-8 cells, the cell line that has most frequently
4 been used for studying *Cryptosporidium* in cell culture so far (Hijjawi 2010, Karanis 2017),
5 could not be used to successfully infect subsequent cell cultures. These findings are in
6 accordance with previous studies showing that *Cryptosporidium*-infected HCT-8 cultures did
7 not produce enough infectious oocysts to maintain infected cultures (Hijjawi 2010), which
8 had also raised concerns about the suitability of HCT-8 for studying *Cryptosporidium* biology.
9 In addition, *C. parvum*-infected COLO-680N cultures remained productive for substantial
10 length of time (~60 days) resulting in a high yield of oocysts. Using a range of
11 methodologies, we have previously shown that COLO-680N could support the life cycle of *C.*
12 *parvum* and subsequent production of oocysts (Miller et al. 2018a). These methodologies
13 include immunofluorescence microscopy, electron microscopy, PCR-based detection of *C.*
14 *parvum* DNA, qPCR in infected cell lines, and lipidomics fingerprinting (Miller et al. 2018a).
15 Some of these methods will be described in detail in the protocols below. Hence, we
16 discovered the first easy-to-handle cell culture system for *Cryptosporidium* that enables the
17 sustainable production of *Cryptosporidium* oocysts at a laboratory scale and the systematic
18 study of the parasite's life cycle and biology, including the identification of potential drug
19 targets.
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33 The follow-up procedure describes a set of protocols and methods for culturing and
34 maintaining *Cryptosporidium* in COLO-680N cell culturing systems along with a description
35 of a range of methods of semi-quantitative and qualitative assessment of *de novo* produced
36 oocysts. These include western Blot analysis with anti-L23-A, PCR, immunofluorescence with
37 L23-A (Miller et al. 2018b). Each of these protocols could be followed separately, or used in
38 series as described below.
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46 **Safety procedures**

47
48 All experimental procedures should be performed in a category 2 containment level
49 laboratory with subsequent use of good laboratory practice. Individuals handling the
50 parasites should always wear appropriate personal protective equipment (lab coat, gloves
51 and goggles) and keep a spill kit in the lab. To avoid potential interaction/infection with
52 *Cryptosporidium*, the culturing and manipulation of the parasite should take place in a
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3 restricted class II biosafety cabinet using aseptic technique. Upon completion of each
4 experiments, the area should be disinfected with 6 % hydrogen peroxide and allow 20
5 minutes for disinfectant contact time.
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8
9

10 11 **Purchasing and handling *Cryptosporidium* oocysts**

12
13 *Cryptosporidium parvum* oocysts can be purchased either from Moredun (e.g. *C.*
14 *parvum* Moredun Strain, Scotland, UK) or from Bunch Grass Farm (*C. parvum* Iowa strain;
15 Idaho, USA). The oocysts were stored in 4 °C for up to six months in 1 X PBS buffer with 1
16 unit/mL penicillin and 1 µg/mL streptomycin, final concentration.
17
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23 **CRYPTOSPORIDIUM CULTURING IN COLO-680N**

24
25 COLO-680N is a good platform for culturing *C. parvum* as it is readily infected and
26 supports the full life cycle of the parasite (Miller et al. 2018a). While presence of *C. parvum*
27 intracellularly appears to be lower in COLO-680N compared to other reference cellular
28 model such as HCT-8, cells can survive the infection for a longer period and oocyst
29 production can be sustained for periods exceeding two weeks (in standard culture flasks) or
30 over a month (in a miniPERM® SM bioreactor, see production of *de novo* oocysts from
31 COLO-680N infected cells in a miniperme® SM bioreactor). Here, we detail the procedure to
32 carry out infection of COLO-680N by *C. parvum* and maintain COLO-680N cell lines in routine
33 format.
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42

43 **Materials**

44 COLO-680N, human oesophageal squamous-cell 3 carcinoma, obtained from CLS Cell Line
45 Services, Eppelheim, Germany
46

47 COLO-680N medium (see REAGENTS AND SOLUTIONS), pre-warmed at 37 °C.
48

49 70 % ethanol
50

51 Oxoid phosphate-buffered saline (DulbeccoA) tablets (ThermoFischer Scientific, cat. no.
52 BR0014G, pH=7.3).
53

54 0.25 % Trypsin-EDTA, (ThermoFisher Scientific, cat.no. 25200056)
55
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1
2
3 *Cryptosporidium* oocysts (available from Bunchgrass Farm at 2×10^7 oocysts/ mL, 50 mL)

4 Sodium hypochlorite, 5 % chlorine reagent (ACROS Organics, cat.no. 7681-52-9)

5
6 Ice

7
8 37 °C water bath

9
10 15-mL centrifuge tubes

11
12 25-cm² T- flasks

13
14 37 °C incubator maintained at 5 % CO₂

15
16 Haemocytometer or cell counter

17
18 1.5-mL microcentrifuge tubes

19
20 *Note: Cryptosporidium* oocysts should be maintained on ice or at 4 °C at all times.

21
22 ***Starting culture from frozen stock of COLO-680N***

- 23
24 1. Store COLO-680N cells at 5×10^6 to 1×10^7 cells per cryovial in COLO-680N medium,
25 10 % DMSO at -80 °C or for long-term storage, in liquid nitrogen.
26
27 2. Quickly thaw the cells (< 1 minute) by gently swirling the vial in the 37 °C water bath,
28 until there is just a little bit of ice left in the vial.
29
30 3. Transfer the vial in the biosafety cabinet and wipe the outside of the vial with 70 %
31 ethanol before opening.
32
33 4. Add cell suspension dropwise to a pre-warmed 15-mL tube, containing 9.0 mL COLO-
34 680N medium.
35
36 5. Centrifuge for 3 min at 1,000 rpm, room temperature.
37
38 *Note: This step removes DMSO.*
39
40 6. Remove the supernatant and resuspend the cells in 10 mL COLO-680N medium.
41
42 7. Add cells to a 25-cm² flask and place in an incubator at 37 °C with 5 % CO₂ until 80 –
43 90 % confluent (typically 1 to 2 days, depending on initial titre).
44
45 8. Remove the culture medium and rinse the cells with 10 mL phosphate-buffered
46 saline (PBS).
47
48 9. Add 1 mL trypsin 0.25% to the flask, swirl liquid ensuring all the growth area is
49 covered before replacing in the incubator for 3 – 5 min or until COLO-680N cells have
50 become detached.
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1
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3 *Note:* COLO-680N are hard to detach and we recommend 0.25 % trypsin rather than
4 0.05 % (as this otherwise results in prolonged exposure to trypsin, in the absence of
5 medium, which is detrimental to the cells).
6
7

- 8 10. Resuspend the cells in 10 mL COLO-680N medium to neutralize trypsin.
9
10 11. Take 100 µl to perform cell counting using the trypan blue viability dye assay and a
11 haemocytometer.
12
13 12. In the meantime, spin down the cells for 3 min at 1,000 rpm, room temperature and
14 resuspend the pellet in 10 mL COLO-680N.
15
16 13. Seed 10% of cells in a new flask (1 mL if using T25 flasks, 2 mL if using T75 flasks, etc)
17 and top up to desired volume of COLO-680N media (9 mL and 18 mL, respectively).
18 *Note:* COLO-680N must not be split more than 1:10 as the seeding density will be too
19 low for the cells to resume their normal growth regime.
20
21 14. Passage/ sub-culture cells once more before using for experimental set up.
22 *Note:* This ensures that cell have well adapted to their new environment after revival
23 and enables to expand the culture and achieve a higher titre of cells.
24
25 15. Seed COLO-680N cells at roughly 70% confluency if infecting the next day (please
26 refer to **Table 1** for recommended cell number, depending on culturing format).
27
28
29
30
31
32
33

34 ***Infect COLO-680N with Cryptosporidium***

35
36

- 37 16. Transfer the desired number of oocysts to a 1.5 mL tube (as a guide, use **Table 1**,
38 depending on MOI and culturing format).
39 For example, for a T25 format and a MOI=2, transfer 45 µl per infection
40 (corresponding to 0.9×10^6 oocysts or 3.6×10^6 sporozoites).
41
42 17. Centrifuge for 8 min at 2, 000 g and remove supernatant.
43
44 18. In the meantime, prepare fresh excystation solution (see REAGENTS AND
45 SOLUTIONS).
46
47 19. Resuspend spun oocysts in 1 X PBS (original volume).
48
49 20. Incubate in excystation solution for 3 hours at 37 °C, with occasional gentle mixing.
50
51 21. Centrifuge for 8 min at 2,000 g and remove supernatant. Wash pellet once with 1 X
52 PBS.
53
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3 22. Resuspend in 100 μ l COLO-680N medium and dispense in ready-to-infect cell
4 cultures.
5

6 23. Intracellular stages of infection can be detected from by fluorescence microscopy
7 from Day4 post-infection and *de novo* oocysts can be isolated from Day 6 post-
8 infection onwards.
9

10
11 *Note: de novo* oocysts can be detected from Day 4 but production peaks at Day 9 –
12 10 (summative effect).
13
14
15
16
17

18 **PRODUCTION OF *DE NOVO* OOCYSTS FROM COLO-680N INFECTED CELLS IN A MINIPERM[®]** 19 **SM BIOREACTOR**

20
21 A long-sought after feature in the field of cryptosporidiosis research has been the
22 development of an *in vitro* system, capable of supporting infection of *C. parvum* (or other
23 species). Having a system that can do so for any period of time, would be ideally suited to
24 further characterise its complex biology of infection. Earlier reports demonstrated that *C.*
25 *parvum* is amenable to genetic manipulation (Pawlowic et al. 2017, Vinayak et al. 2015), and
26 could be tractable. However, such approach is constrained by the survival of the host. COLO-
27 680N cells remain fit following infection by *C. parvum* and as a result our lab has been able
28 to establish a long-term culture system, continuously yielding oocysts, paving the way for
29 more *ex-vivo* research (see **Figure 1**). There are currently several existing commercially
30 available platforms that are suitable for large scale culturing (including CELLline adhere
31 bioreactor flasks, Sigma-Aldrich, cat. no. Z688045; miniPERM[®] SM bioreactor, Sarstedt, cat.
32 no. 94.6077.618). Here, we describe our recent methodology and results using the
33 miniPERM bioreactor system.
34
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46 **Materials**

47 COLO-680N cells

48 COLO-680N medium (see REAGENTS AND SOLUTIONS), pre-warmed at 37 °C.

49 70 % ethanol

50 Oxoid phosphate-buffered saline (DulbeccoA) tablets (ThermoFischer Scientific, cat. no.
51 BR0014G, pH=7.3).

52 0.25 % Trypsin-EDTA (ThermoFisher Scientific, cat.no. 25200056)
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1
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3 miniPERM® SM bioreactor (Sigma-Aldrich, cat. no. Z688045)

4 *Cryptosporidium* oocysts (available from Bunchgrass Farm at 2×10^7 oocyst/ mL, 50 mL)

5 Sodium hypochlorite, 5 % chlorine reagent (ACROS Organics, cat.no. 7681-52-9)

6 Haemocytometer

7
8
9 Ice

10
11
12
13 **Protocol**

- 14 1. Expand COLO-680N cell culture so that enough cells are ready for seeding the
15 bioreactor (usually two T175 flat flasks).
- 16 2. Detach cells and isolate 36.8×10^6 cells.
- 17 3. Resuspended in 35 mL with COLO-680N medium, supplemented with amphotericin B
18 x1000 (stock at 0.2 µg/ mL).
- 19 4. Draw cells into a 50 mL syringe and inoculate into the production module of the
20 bioreactor through the sample port (**Figure 1A**).
- 21 5. Add 400 mL of warm culture media, supplemented with amphotericin B, to the
22 nutrient module.
- 23 6. Place the bioreactor on a roller (SLS Lab Basics) in an incubator.
- 24 7. Maintain at 37 °C, 5 % CO₂ at a speed of 10 rpm.
- 25 8. After 24 hours, prepare oocysts as described in *C. parvum* infection at a MOI of 2 (18
26 $\times 10^6$ oocysts), with excysted sporozoites being resuspended in 1 mL of 1 X PBS.
- 27 9. Remove 1 mL of culture media from the production module and inoculate the
28 sporozoite suspension into the production module, through the sample port, before
29 returning to an incubator.
- 30 10. Once a week, remove medium from the nutrient module and replace with fresh
31 medium- warmed to 37 °C.
- 32 11. Remove ½ volume from the production module to collect the oocysts and replace
33 with same volume of fresh medium.
- 34 12. Clear oocysts from cell debris by centrifuging at 300 g for 3 minutes.
- 35 1. Transfer supernatant. Collect supernatant and further spin at 5,000 g for 8 min.
- 36 13. Resuspend in 200 µl 1 X PBS, with 1 unit per mL penicillin and 1 µg/mL streptomycin
37 final concentration.
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14. Oocysts can be counted using a haemocytometer, under magnification x400. Starting at 1/100 dilution.
15. Store at 4 °C.

DETECTION of *C. parvum* OOCYSTS USING IMMUNO-BASED ASSAYS (L23-A antibodies)

Current immuno-based assays for detecting *C. parvum* oocysts produced *in vitro* rely on products from Waterborne, INC (Crypt-a-Glo, cat.no. A400FLR-1 X, Sporo-Glo, cat.no. A600FLR-1 X). While these reagents are highly performing for the detection of Cryptosporidia in environmental and clinical samples, they are limited to fluorescence-based assessment of *Cryptosporidium* infection.

Antibodies targeted to *C. parvum* 60S ribosomal subunit L23-A, have previously been described by the authors (Miller et al. 2018b) and are suitable for western blot analysis and immunofluorescence **Figure 2A** and **Figure 3**, respectively.

*Note: L23-A antibodies described here are also suitable for detection of *C. ubiquitum* and *C. hominis* as the peptide sequence used for immunisation (Miller et al. 2018b) shares 100% identity between the three species.*

Detection of *C. parvum* oocysts by western blot analysis (L23-A antibodies)

Western Blot Analysis of L23-A can be used to verify the presence of *de novo* produced oocysts in COLO-680N (or alternative platform for *C. parvum* -host co-culture) but also allows semi-quantitative analysis (see **Figure 2A**, left and right panels).

Materials

C. parvum oocysts

Lysis buffer (see REAGENTS AND SOLUTIONS)

5 X SDS loading buffer (SEE REAGENTS AND SOLUTIONS)

15% SDS-PAGE gel

0.2 µm PVDF blotting membrane (GE Healthcare Life Sciences, cat. no. 10600021)

L23-A primary antibody [Eurogentech, custom-designed; (Miller et al. 2018b)]

HRP-conjugated secondary antibodies (Sigma-Aldrich, cat.no. A9046).

1
2
3 Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences, cat.
4 no. RPN2232)

5
6 Amersham Hyperfilm ECL (GE healthcare Life Sciences, cat.no. 28906835)
7
8

9
10 **Protocol**

- 11 1. Resuspend 2×10^6 *C. parvum* oocysts in 160 μ l lysis buffer.
- 12 2. Sonicate at 14 watts/cm², 3 x 30 seconds to break down the oocyst envelope and
13 shear the DNA.
14
- 15 3. Add 40 μ l of 5 X SDS loading buffer.
16
- 17 4. Load 10 μ l of total protein on 15 % SDS-PAGE gels
18
- 19 5. Transfer onto PVDF membrane.
20
- 21 6. Probe with L23-A primary antibody (Miller et al. 2018b) overnight and HRP-
22 conjugated secondary antibody for 1 hour (see **Figure 2A**, left and right panels).
23
- 24 7. Catalyse the peroxidase reaction using ECL prime reagent.
25
- 26 8. Develop the blot on an x-ray film.
27
28

29 *Note:* This methodology enables the detection of $< 10^5$ oocysts per well and allow
30 quantitative analysis of oocysts over a $10^5 - 10^7$ range. As sensitivity is dependent on
31 individual antibody batch, establishing the linear range of the signal requires to conduct
32 dilutional experiments beforehand. We recommend starting between 1:500 and 1: 3000 for
33 primary antibodies and 1:10000 and 1:20000 for secondary antibodies, when using 10^5
34 oocysts and over.
35
36
37
38
39
40
41

42 ***Detection of C. parvum oocysts by immunofluorescence (L23-A antibodies)***

43
44 This methodology can be used for qualitative assessment of oocysts in different
45 experimental setups and offers a good alternative to Crypt-a-Glo (Waterborne INC, cat.no.
46 A400FLR-1 X), see **Figure 3**.
47
48
49

50
51 **Materials**

52 *C. parvum* oocysts

53 15-mL centrifuge tubes

54 Microscope slides
55
56
57
58
59
60

1
2
3 Coverslips

4 Poly-L lysine solution (Sigma-Aldrich, P4707)

5
6 Fixative reagent (see REAGENTS AND SOLUTIONS)

7
8 0.5 % Triton-X100

9
10 Oxoid phosphate-buffered saline (DulbeccoA) tablets, (ThermoFischer Scientific, cat. no.
11 BR0014G, pH=7.3).

12
13 Goat serum (Sigma-Aldrich, cat. no. G9023)

14
15 L23-A primary antibody (Miller et al. 2018b)

16
17 AlexaFluo488 labelled secondary antibodies (for immunofluorescence, Life Technologies,
18 cat.no. A11039).

19
20 Fluoroshield histology mounting medium with DAPI (Sigma-Aldrich, cat.no. F6057)

21
22
23 **Protocol**

- 24
- 25 1. Transfer oocysts-containing supernatants from infected flasks in 15-mL centrifuge
26 tubes.
 - 27
 - 28 2. Spin for 3 min at 300 g.
29 *Note:* This step is required to clear cell debris present in the supernatant.
 - 30
 - 31 3. Collect supernatant and further spin at 5,000 g for 8 min.
 - 32
 - 33 4. Resuspend in 20 – 100 μ l 1 X PBS depending on size of pellet and leave on ice until
34 ready to use.
 - 35
 - 36 5. Carry out a short excystation procedure (1 hour) following the protocol described
37 earlier (Infect COLO-680N with *Cryptosporidium*, steps 16-12), adapting volume as
38 desired.
 - 39
 - 40 6. Coat microscopy slides with poly-L lysine diluted at 0.1 mg/mL and leave for 10 min,
41 room temperature, drain slides and dry them in a 60 °C oven for 1 hour.
 - 42
 - 43 7. Add one drop of oocyst suspension on microscopy slide and leave to dry at room
44 temperature.
 - 45
 - 46 8. Add a drop of fixative reagent and leave for 10 min at room temperature.
 - 47
 - 48 9. Wash once with 1 X PBS.
 - 49
 - 50 10. Add ice-cold 0.5 % triton X-100, for 5 min.
 - 51
 - 52 11. Wash once in 1 X PBS.
 - 53
 - 54 12. Incubate with 3 % goat serum in 1 X PBS for 30 min at room temperature.
 - 55
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 - 60

13. Drain solution and place in the microslide holder of a staining box. Leave overnight in primary antibody, at 1/500 in 3 % goat serum, 1 X PBS.
14. Wash three times in 1 X PBS.
15. Add secondary antibody, at 1/1000 in 3% goat serum, 1 X PBS for 1 hour at room temperature.
16. Wash three times in 1 X PBS.
17. Add one drop of mounting medium with DAPI and add a coverslip.
18. Remove any excess liquid very carefully with tissue and seal with nail polish.

DETECTION of *C. parvum* OOCYSTS USING Polymerase Chain Reaction (PCR)

This methodology can be used to estimate *C. parvum* oocysts shed from animals or produced *in vitro*, see **Figure 2 B**.

Materials

Purified *C. parvum* oocysts (control, 2×10^6 oocysts in 100 μ l 1 XPBS)

Dry ice

50 °C waterbath

1.5 mL Eppendorf tubes

0.2 mL thin-walled PCR tubes

Thermal Cycler

FastStart DNA polymerase, dNTPs (Roche, cat.no. 04738381001)

Forward Primer: 5' GCTGGTGATACTCACTTGGGTGGTG 3'

Reverse Primer: 5' CTCTTGCCATACCAGCATCCTTG 3'

Sterile water

Agarose

1 X TAE (Tris Acetic acid EDTA) or TBE (Tris borate EDTA) buffer

Protocol

1. Prepare a crude oocyst extract by freezing/ thawing the oocyst suspension, three times.
2. Prepare dilution 1:10, 1:100, 1:1000, 1:10000 of crude oocyst extracts.

- 1
- 2
- 3 3. Prepare a master mix (x reactions), as described in **Table 2**, excluding the crude
- 4 oocyst extracts.
- 5
- 6 4. Aliquot master mix in PCR tubes (20 μ l).
- 7
- 8 5. Add crude oocysts extract (5 μ l).
- 9
- 10 6. Run thermal cycling programme (95°C for 3 min; 95°C for 30s, 52°C for 30s, 72°C for
- 11 30s, for 40 cycles).
- 12
- 13 *Note:* The final extension is not required for this procedure.
- 14
- 15 7. Load 10 μ l onto 1.2 % agarose gel in 1 XTAE (or 1 X TBE) buffer.
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20 REAGENTS AND SOLUTIONS

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22 *We use HPLC water in all solutions*

23 24 25 26 27 **COLO-680N medium**

28
29 For 500 mL medium: 440 mL RPMI-1640 with L-glutamine and sodium bicarbonate (MERCK,
30 cat.no. R8758), 50 mL heat-inactivated Foetal Bovine Serum (FBS) (MERCK, cat.no. F7524),
31 10 mL Penicillin-Streptomycin solution at 1000 units/mL (ThermoFisher Scientific, cat.no.
32 15140122).
33
34
35

36 37 38 39 **Excystation medium**

40
41 To 400 μ l 0.5 % sodium hypochlorite, add 100 μ l 0.05 % Trypsin-EDTA. Scale up if necessary.
42
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44
45

46 47 **Lysis buffer**

48
49 20 mM Tris-Cl (pH 7.5), 10 mM EDTA, 10 mM EGTA, 150 mM NaCl and 1 % (w/v) Triton and
50 one tablet of protease inhibitor cocktail (Roche).
51
52
53
54

55 56 **5X SDS loading buffer**

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3 5% β -mercaptoethanol, 0.02 % bromophenol blue, 30 % glycerol, 10 % sodium dodecyl
4 sulphate, 250 mM, pH 6.8]
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9 ***Fixative solution (to be prepared in a fume hood)***
10

11 For 100 mL of fixative, dissolve 3g paraformaldehyde in 90 mL H₂O and place on heated
12 plate (with stirring) set at 60 °C. Add a few drops of concentrated NaOH to help dissolve the
13 paraformaldehyde. Once dissolved, add 1 mL 25% glutaraldehyde, 2.5 mL 10% Triton X-100.
14 Check the pH is around neutral and make up the volume to 100 mL. The fixative can be
15 aliquoted and stored at -20 °C.
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25 **ACKNOWLEDGEMENT**
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37 **LITERATURE CITED**
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49 50 51 52 **FIGURE LEGENDS**

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55 **Figure 1: Production of *de novo* oocysts from COLO-680N infected cells in a miniperme® SM**
56 **bioreactor. A.** Schematic diagram of bioreactor with its two main parts: the nutrient module

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2
3 and the production module. Cells and oocysts are seeded in the production module via the
4 sample port, using a syringe as shown in the diagram. **B.** A bar chart representing the
5 oocysts production per mL, per month in the bioreactor. Total volume in production module
6 is 35 – 40 mL.
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12 **Figure 2: Semi-quantitative analysis of *C. parvum* oocysts using western blotting and**
13 **qPCR. A.** Western blot analysis against L23-A antibody (1st), with various concentrations of
14 primary and anti-HRP secondary antibodies (2nd). Left panel: 5 µl of undiluted and 1:10
15 dilution of total proteins were loaded (equivalent to 1.25×10^6 and 1.25×10^5 oocysts,
16 respectively). Right panel: from 1.25×10^5 oocysts: **i.** Primary 1:3,000, Secondary 1:20,000;
17 **ii.** Primary 1:3,000, Secondary 1:50,000; **iii.** Primary 1:5,000, Secondary 1:20,000; **iv.** Primary
18 1:5,000, Secondary 1:50,000. **B.** PCR analysis using specific anti-*Cryptosporidium* Hsp70
19 primers. Left panel: Control (Bunch Grass Farm purified oocysts), 1 (first round of *de novo*
20 produced oocysts from COLO-680N infected cells); Right panel: 1.1, 1.2 and 1.3 correspond
21 to *de novo* produced oocysts by COLO-680N cells infected with oocysts from the first round
22 of infection (1).
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34 **Figure 3: Immunofluorescent detection of oocysts.** L23-A antibody (Miller et al. 2018b) and
35 AlexaFluor 594 secondary (red) antibodies along with DAPI (blue) were used to detect
36 oocysts produced in calves or *in vitro*. Differential interference contrast (DIC) was used to
37 enhance the contrast in unstained samples (oocysts). “Merged 1” includes L23-A
38 colocalization with DAPI. “Merged 2” includes L23-A colocalization with DIC. Scale bar: 10
39 µm.
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TABLES

Table 1: Recommended COLO-680N cell and *C. parvum* oocyst number for infection, depending on experimental format.

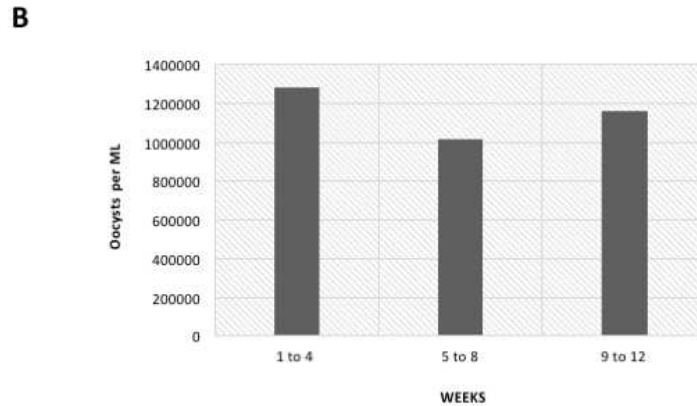
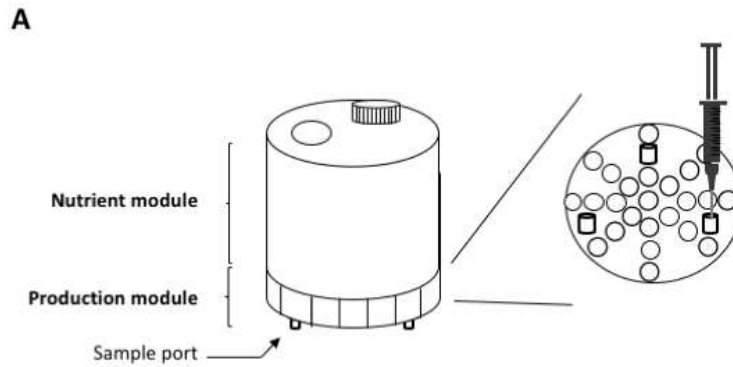
Format	Growth area (cm ²) *	Estimate Cell Number at 70% confluency	Multiplicity of Infection (MOI) & Volume of oocyst suspension (μl)**		
			0.5	2	5
96-well	0.32	0.23 x 10 ⁵	0.3	1.20	3
48-well	0.95	0.68 x 10 ⁵	0.4	1.75	4
24-well	1.90	1.35 x 10 ⁵	0.9	3.5	9
6-well	9.5	6.70 x 10 ⁵	4	17.0	43
T25	25	1.75 x 10 ⁶	11	45	113

* Based on information with Corning products

** Stock solution is 10⁹ oocysts/ 50 mL in 1 X PBS (Pen/Strep), BunchGrass Farm (*C. parvum* type IIa isolate. IOWA).

Table 2: Reaction mix for semi-quantitative analysis of oocysts by PCR

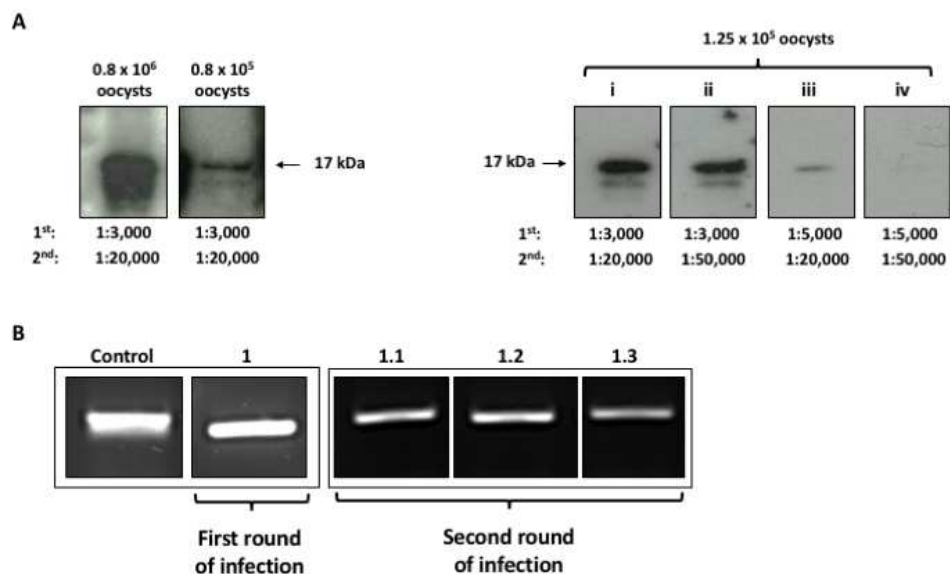
	1 X Reaction (25 μl)
Crude oocysts extract (1:10, 1:100, 1:1000, 1:10000)	5 μl
FastStart Taq DNA polymerase buffer (Roche)	2.5 μl
F primer (100 pmoles/μl)	0.2 μl
R primer (100 pmoles/μl)	0.2 μl
dNTPs (10 mM)	0.5 μl
Taq DNA polymerase (5U/μl)	0.2 μl
DNase-free water	16.4 μl



Production of de novo oocysts from COLO-680N infected cells in a miniperm® SM bioreactor. A. Schematic diagram of bioreactor with its two main parts: the nutrient module and the production module. Cells and oocysts are seeded in the production module via the sample port, using a syringe as shown in the diagram.

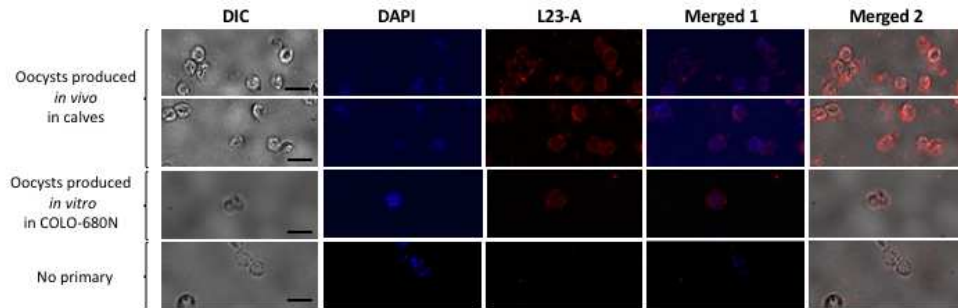
B. A bar chart representing the oocysts production per mL, per month in the bioreactor. Total volume in production module is 35 – 40 mL.

254x254mm (72 x 72 DPI)



Semi-quantitative analysis of *C. parvum* oocysts using western blotting and qPCR. A. Western blot analysis against L23-A antibody (1st), with various concentrations of primary and anti-HRP secondary antibodies (2nd). Left panel: 5 μ l of undiluted and 1:10 dilution of total proteins were loaded (equivalent to 1.25×10^6 and 1.25×10^5 oocysts, respectively). Right panel: from 1.25×10^5 oocysts: i. Primary 1:3,000, Secondary 1:20,000; ii. Primary 1:3,000, Secondary 1:50,000; iii. Primary 1:5,000, Secondary 1:20,000; iv. Primary 1:5,000, Secondary 1:50,000. B. PCR analysis using specific anti-Cryptosporidium Hsp70 primers. Left panel: Control (Bunch Grass Farm purified oocysts), 1 (first round of de novo produced oocysts from COLO-680N infected cells); Right panel: 1.1, 1.2 and 1.3 correspond to de novo produced oocysts by COLO-680N cells infected with oocysts from the first round of infection (1).

254x175mm (72 x 72 DPI)



Immunofluorescent detection of oocysts. L23-A antibody (Miller et al. 2018b) and AlexaFluor 594 secondary (red) antibodies along with DAPI (blue) were used to detect oocysts produced in calves or in vitro. Differential interference contrast (DIC) was used to enhance the contrast in unstained samples (oocysts). "Merged 1" includes L23-A colocalization with DAPI. "Merged 2" includes L23-A colocalization with DIC. Scale bar: 10 μ m.

338x190mm (54 x 54 DPI)